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A Kinetic Method for Serum 5'-Nucleotidase Using Stabilised Glutamate Dehydrogenase

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Summary: A modification of a kinetic determination of 5'-nucleotidase (EC 3.1.3.5) activity is described. Special attention has been paid to the stabilisation of glutamate dehydrogenase (EC 1.4.1.2) by *L*-leucine, optimal NADH concentration and the influence of endogeneous ammonia. The optimal concentrations of the other constituents of the reagent were checked with the optimal values given in the literature. Normal values were determined. The proposed method shows a good correlation with a colorimetric reference method.

Ein kinetisches Verfahren zur Bestimmung von 5'-Nucleotidase im Serum unter Verwendung stabilisierter Glutamatdehydrogenase

Zusammenfassung: Die Modifikation einer kinetischen Bestimmung von 5'-Nucleotidase (EC 3.1.3.5) wird beschrieben. Besonders wurde auf die Stabilisierung der Glutamatdehydrogenase (EC 1.4.1.2) durch *L*-Leucin, optimale NADH-Konzentration und den Einfluß von endogenem Ammoniak geachtet. Die optimalen Konzentrationen der übrigen Bestandteile des Reagenz wurden mit den in der Literatur angegebenen optimalen Werten überprüft. Die vorgeschlagene Methode zeigt eine gute Korrelation mit einer kolorimetrischen Bezugsmethode.

Introduction

Recently, kinetic methods for the determination of 5'-nucleotidase have been described using bovine glutamate dehydrogenase in a coupled assay (1, 2). Briefly, adenosine liberated by the action of 5'-nucleotidase is converted to inosine and ammonia with adenosine deaminase (EC 3.5.4.4). In a coupled reaction catalysed by glutamate dehydrogenase ammonia forms glutamate in the presence of 2-oxoglutarate and NADH. The overall reaction is monitored by the decrease in extinction at 340 nm caused by conversion of NADH to NAD. A fairly high concentration of NADH is required to compensate for side reactions such as removal of endogeneous ammonia and pyruvic acid during a pre-incubation period. NADH however, will inactivate glutamate dehydrogenase especially at higher concentrations (3). The inactivation promoted by NAD can be abolished by adding allosteric modifiers such as ADP (3, 4, 5) or *L*-leucine (6, 7). Preliminary experiments revealed that *L*-leucine, unlike ADP (1), does not affect the measurement of 5'-nucleotidase activity (*Persijn*, personal communication). The present report describes a kinetic assay with glutamate dehydrogenase protected against inactivation under conditions optimal for the measurement of 5'-nucleotidase activity.

Materials and Methods

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The enzyme reaction rates were measured at 340 nm on a Photovolt Era I reaction rate analyzer at 37°C. The following solutions were prepared:

Solution 1: Dissolve in 900 ml aqua dest. 17.28 g triethanolamine/HCl (0.115 mol/l), 3.02 g *L*-leucine (23 mmol/l), 847 mg Na-2-oxoglutarate (5.75 mmol/l) and 12.53 g Na-β-glycerophosphate (57.5 mmol/l). Adjust to pH 7.6, add 259 mg $MnSO_4 \cdot 2H_2O$ (1.15 mmol/l) and dilute to 1000 ml.

Stable for 2 weeks at 4°C.

Solution 2: Dissolve 144 mg NADH (40 mmol/l) in 5 ml aqua dest. Stable for 1 week at 4°C.

Solution 3: Adenosine deaminase in glycerol, 400 kU/l, Boehringer 15069 EAAT.

Solution 4: Glutamate dehydrogenase in glycerol, 900 kU/l, Boehringer 15324 EGAH.

Solution 5: Dissolve 10 mg 5'-AMP (23 mmol/l) in 1 ml water. Prepare fresh daily.

Working solution: Prior to use, mix solutions 1, 2, 3 and 4 in the ratio 10 ml + 0.1 ml + 0.01 ml + 0.1 ml. Stable for 4 hr at room temperature. The final concentrations in the cuvet are according the selected values of table 1.

Procedure

Pipet 0.2 ml of fresh serum into a test tube and add 2 ml working solution, mix and incubate the tube for 30 min in a waterbath at 37°C. Add then 0.1 ml of solution 5 and mix. Measure the ΔE/min after 2 min temperature equilibration.

Tab. 1. Optimal concentrations of reagents in the cuvet.

Reagent	Range	Selected
L-leucine	20–30 mmol/l	20 mmol/l
NADH	0.15–0.45 mmol/l	0.35 mmol/l
2-oxoglutarate	3–10 mmol/l	5 mmol/l
5'-AMP	0.75–3 mmol/l	1 mmol/l
Glutamate dehydrogenase	5–13 kU/l	9 kU/l
adenosine deaminase	0.1–0.5 kU/l	0.4 kU/l
triethanolamine/HCl	not studied	100 mmol/l
β-glycerophosphate	not studied	50 mmol/l
Mn^{++}	not studied	1 mmol/l

To obtain the activity in U/l, multiply the ΔE/min by the factor 1850. This factor is calculated as follows:

$$\text{Factor} = \frac{1000 \times V_t}{\epsilon \times V_s}$$ V_t= total voume, V_s = sample volume

and ϵ is the molar absorption coefficient of NADH at the wave length used (6.22 $cm^2/\mu mol$).

Results and Discussion

Optimal *L*-leucine concentration

To establish the optimal concentration of *L*-leucine for the activity of glutamate dehydrogenase, the following experiment was carried out. Five working solutions were prepared with *L*-leucine concentrations of 5, 10, 15, 20, and 30 mmol/l. Five min after reagent preparation glutamate dehydrogenase activity was measured at 340 nm after adding 0.07 μmol NH_4Cl to 2 ml aliquots of the working solution. According to figure 1 a *L*-leucine concentration of 20 mmol/l and more is optimal. This result disagrees with the literature where a *L*-leucine concentration of 10 mmol/l is already optimal (4, 7). An explanation of this different result is the high NADH concentration in the working solution. In the proposed method: 0.35 mmol/l, *Persijn* et al (7): 0.19 mmol/l, *Jung* et al (4): 0.25 mmol/l. A *L*-leucine concentration of 20 mmol/l was chosen in the proposed method and used in further experiments.

Glutamate dehydrogenase stabilisation

To evaluate the glutamate dehydrogenase stabilising effect of *L*-leucine, and the influence of Mn^{++}, working solutions containing all reagents in their optimal concentration (sol. A), with Mn^{++} (1 mmol/l) and no *L*-leucine (sol. B) and no *L*-leucine and Mn^{++} (sol. C) were prepared and allowed to age for 60 min at 25 °C. Directly following reagent preparation and then at 15 min intervals, glutamate dehydrogenase activity was assayed by adding 0.07 μmol NH_4Cl to 2 ml aliquots of the solutions and measuring the decrease in absorption at 340 nm during the first min. The results shown in figure 2 indicate a rapid inactivation of glutamate dehydrogenase in the absence of *L*-leucine. Although the presence of Mn^{++} in a concentration of 1 mmol/l slightly inhibits the glutamate dehydrogenase activity (1), it is necessary for activation of 5'-nucleotidase (8, 9). In addition it also has a small stabilising effect on glutamate dehydrogenase. This is in accordance with the findings of *Ellis & Goldberg* (1). When the working solutions were aged at 37 °C essentially the same results were obtained. At this temperature however, sol. A exhibited after 2.5 hr a glutamate dehydrogenase activity of 80% of the initial value. This has no practical consequences because of the broad optimal range of glutamate dehydrogenase activity (tab. 1). Absence of Mn^{++} in solution A in this experiment had no effect on the stability. It is evident that incorporation of *L*-leucine in the working solution contributes considerably to its stability at room and assay temperature.

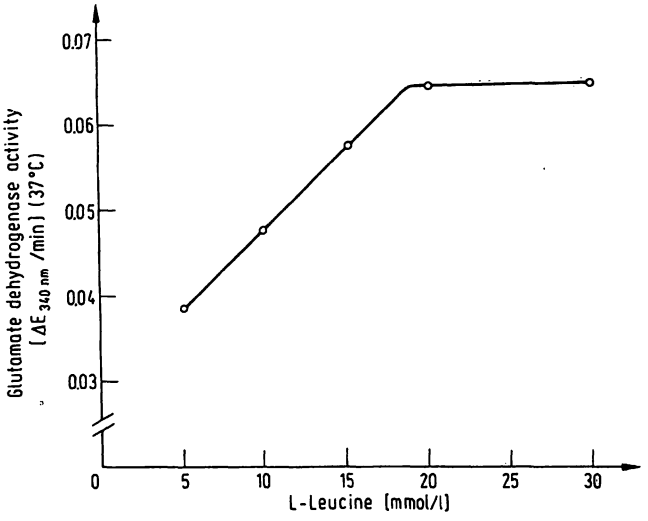

Fig. 1. Effect of different *L*-leucine concentrations on glutamate dehydrogenase activity.

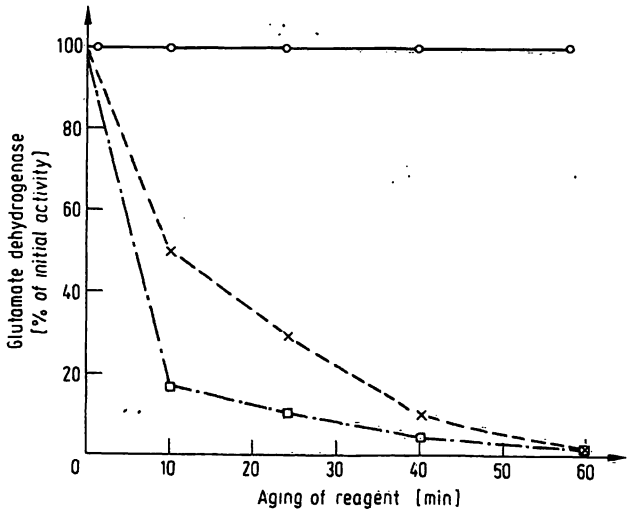

Fig. 2. Effect of *L*-leucine (20 mmol/l) and Mn^{++} (1 mmol/l) on glutamate dehydrogenase stability. Working solutions A, B, and C contained *L*-leucine and Mn^{++} (o——o), no *L*-leucine (x——x) and no *L*-leucine and Mn^{++} (Δ——Δ) resp.

Optimal NADH concentration and effect of endogeneous ammonia

The optimal NADH concentration in the working solution was established with 3 serum samples with a normal, an elevated and a high 5'-nucleotidase activity resp. The NADH concentration ranged from 0.15–0.45 mmol/l (fig. 3), the other constituents had the selected values as given in Table 1. The concentration of 0.35 mmol/l was chosen for the proposed method to ensure an optimal NADH concentration during the assay, even at high endogeneous ammonia levels and, as a consequence, high NADH consumption during the pre-incubation time. Figure 4 illustrates the decrease in absorbance during a pre-incubation of 3 serum samples with 5'-nucleotidase activities of 25, 63 and 125 U/l resp. The serum sample of 63 U/l was enriched with ammonia to a concentration of 250 µmol/l. After a rapid drop in absorbance, initial decrease in absorbance approaches zero after approximately 25 min and corresponds to a 5'-nucleotidase activity of less than 0.5 U/l, even in a sample with a high ammonia concentration. The results indicate that a pre-incubation time of 30 min effectively eliminates side reactions causing non-specific NADH consumption. To demonstrate that after the pre-incubation the NADH concentration is still high enough to carry out the assay, 2 serum samples with different amounts of ammonia were pre-incubated, followed by the determination of the 5'-nucleotidase activity. The results are shown in table 2. It is obvious that ammonia up to a concentration of 250 µmol/l has no effect on the determination of 5'-nucleotidase activity.

Tab. 2. Effect of endogeneous ammonia.

ammonia added [µmol/l]	0	50	125	250
5'-nucleotidase activity [U/l]				
sample A	29	30	29	29
sample B	63	62	62	59

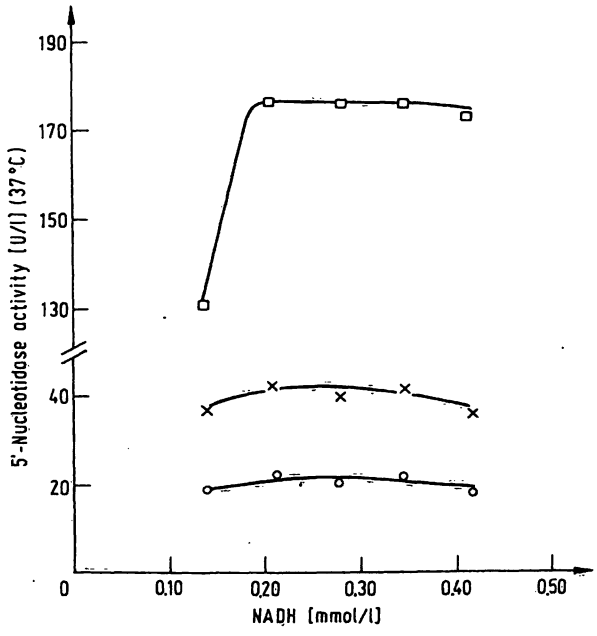

Fig. 3. Optimal *L*-leucine concentration.

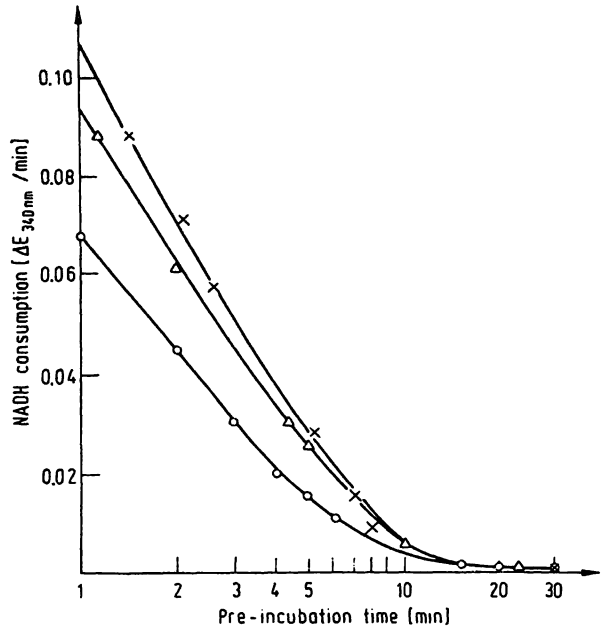

Fig. 4. NADH consumption during pre-incubation. 5'-nucleotidase activity of serum samples: 154 U/l (x——x), 63 U/l (△——△) and 25 U/l (o——o). Sample (△——△) contained 250 µmol added ammonia.

Optimal concentrations of reagents

The optimal concentrations of 2-oxoglutarate, 5'-AMP, glutamate dehydrogenase, adenosine deaminase and the pH value given in the literature were checked in the proposed method. Experiments with sera having a normal and an elevated 5'-nucleotidase activity resp. revealed that maximal and constant activities were obtained when final reagent concentrations in the reaction mixture were within the ranges shown in table 1. These ranges are in good agreement with the optimal ranges given in the literature (1, 2, 4, 7). For the additional reagents Na-β-glycerophosphate, Mn^{++} and the buffer

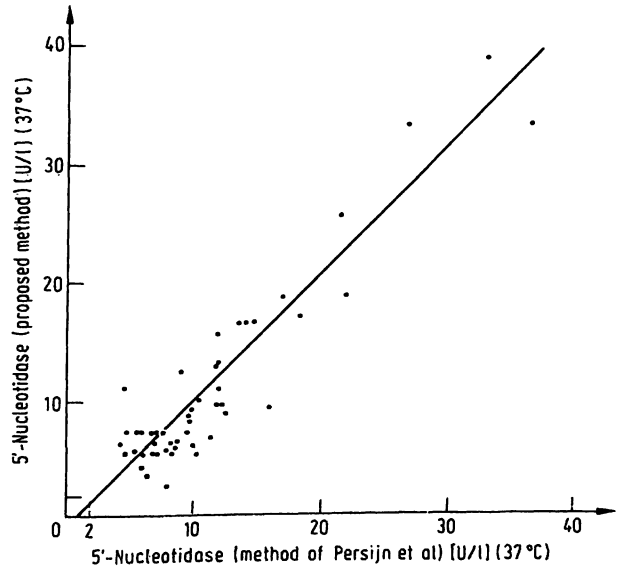

Fig. 5. Comparison of proposed method with reference method.

composition the recommandations of *Ellis & Goldberg* were followed (1). For the routine procedure final concentrations in the cuvet were selected as given in table 1.

Comparison with other method

This method was compared with the technique of *Persijn* et al. (10) by parallel analyses of 51 serum samples. The results are plotted in figure 5. The regression equation ($y = 1.08x - 0.69$, $S_y \cdot x = 4.8$ and the coefficient of correlation is 0.937) indicates a good correlation between the two methods.

Precision and normal values

The interrun precision in the normal range was 6.5% ($\bar{x} = 9.8$, $n = 22$) and in the elevated range 4.9% ($\bar{x} = 23.6$, $n = 22$). The normal range, compiled from values of 35 healthy persons, was found to be 2.1–10.6 U/l (95% confidence limits).

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